Detection of PrP in Extraneural Tissues

K. L. BROWN,* D. L. RITCHIE, P. A. MCBRIDE, AND M. E. BRUCE
Institute for Animal Health, Neuropathogenesis Unit, Edinburgh EH9 3JF, United Kingdom

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ABSTRACT Transmissible spongiform encephalopathies (TSEs) or "prion diseases" are a group of unconventional fatal diseases. TSEs are characterised by the accumulation of a modified form of the normal host glycoprotein, PrP (PrP^c). In the course of infection PrP^c is converted to an abnormally protease resistant form, PrP^{Sc}. The exact nature of the infectious agent responsible for these diseases remains controversial. While there is compelling evidence that TSE agents contain an informational molecule, possibly a nucleic acid, some believe that the infectious agent or "prion" is solely composed of PrP^{Sc}. Nevertheless, PrP is required for TSE pathogenesis, as mice devoid of the PrP gene ($PrP^{-/-}$) remain healthy when challenged with TSE isolates and are unable to replicate infectivity within the central nervous system (CNS) or in other tissues. In recent years immunocytochemistry has been used to pinpoint which cells are associated with abnormal accumulations of PrP, providing important information on the cellular targeting of TSE infection. In uninfected and scrapie-infected mice, PrP protein is found in the CNS and in extraneural tissues such as spleen and lymph nodes. In the peripheral lymphoid system, PrP is associated with follicular dendritic cells that are known to be important for replication of infectivity for at least one TSE strain. This review will focus on current methods for the immunocytochemical detection of PrP in murine extraneural tissues, mainly lymphoid tissues, and will discuss recent findings on the role of the peripheral lymphoid system in TSE pathogenesis. *Microsc. Res. Tech. 50:40–45, 2000.*

INTRODUCTION

Scrapie, a natural disease of sheep and goats, is the most studied member of the TSEs, which also include sporadic and "new-variant" Creutzfeldt-Jakob disease (CJD) in humans and bovine spongiform encephalopathy in cattle. TSEs are characterised by pathological changes in the central nervous system including vacuolation, gliosis, and the accumulation of an abnormal form of the host sialoglycoprotein, PrP.

PrP protein is essential for disease replication as transgenic mice devoid of the PrP gene ($PrP^{-/-}$ mice) are resistant to TSE infection (Bueler et al., 1993; Manson et al., 1994). During the course of infection, the normal cellular form of PrP (PrP^c) is modified to an abnormal protease resistant form (PrP^{Sc}), which differs biochemically from PrP^c Although the accumulation of PrP^{Sc} is a feature of TSE infection, the function of PrP^c remains to be fully defined. Studies in $PrP^{-/-}$mice have demonstrated that PrP may be important for the regulation of circadian rhythms (Tobler et al., 1996) and may play a role in T cell activation (Mabbott et al., 1997).

Although the main consequence of TSE infection is CNS degeneration, the peripheral lymphoid system plays an important role in pathogenesis. Following infection by peripheral (e.g., intraperitoneal or oral) routes, most TSE agents replicate in spleen and lymph nodes prior to neuroinvasion, although there is no overt immune system dysfunction or pathology associated with infection of these tissues. Characterising the cells that support replication in these tissues may indicate potential targets for therapy in the early stages of pathogenesis, for example in "new variant" CJD.

In previous studies, the follicular dendritic cell (FDC) was proposed as a candidate cell based on the association of PrP with this cell type in both normal and scrapie infected mice (McBride et al., 1992) and the failure of ionising radiation to influence disease progression (Fraser and Farquhar, 1987). Studies in severe combined immunodeficient (SCID) mice (Bosma et al., 1983), which lack B and T lymphocytes and mature FDCs, strengthen the possibility that FDCs are important in pathogenesis. SCID mice resist peripheral challenge with scrapie and are unable to replicate the agent in their spleens (Fraser et al., 1996; Lasmezas et al., 1996; O'Rourke et al., 1994). These effects are reversed following bone marrow (bm) grafting, which leads to maturation of FDCs (Fraser et al., 1996). In a recent study (Brown et al., 1999), we used chimaeric models where the immune system was manipulated to produce mice where FDCs but not lymphocytes expressed PrP or vice versa. In these models, replication of the ME7 strain of scrapie only occurred in the presence of mature PrP-expressing FDCs and was not dependent on the presence of PrP-expressing lymphocytes.

As the peripheral lymphoid system is involved in many natural and experimental TSEs, the detection of changes in the distribution or appearance of PrP protein will help us to understand the early events in pathogenesis. This review will focus on current methods for the detection of PrP protein in extraneural tissues, particularly in the peripheral lymphoid system.

*Correspondence to K L Brown, Institute for Animal Health, Neuropathogenesis Unit, Ogston Building, West Mains Road, Edinburgh EH9 3JF, UK E-mail karen.brown@bbsrc.ac.uk

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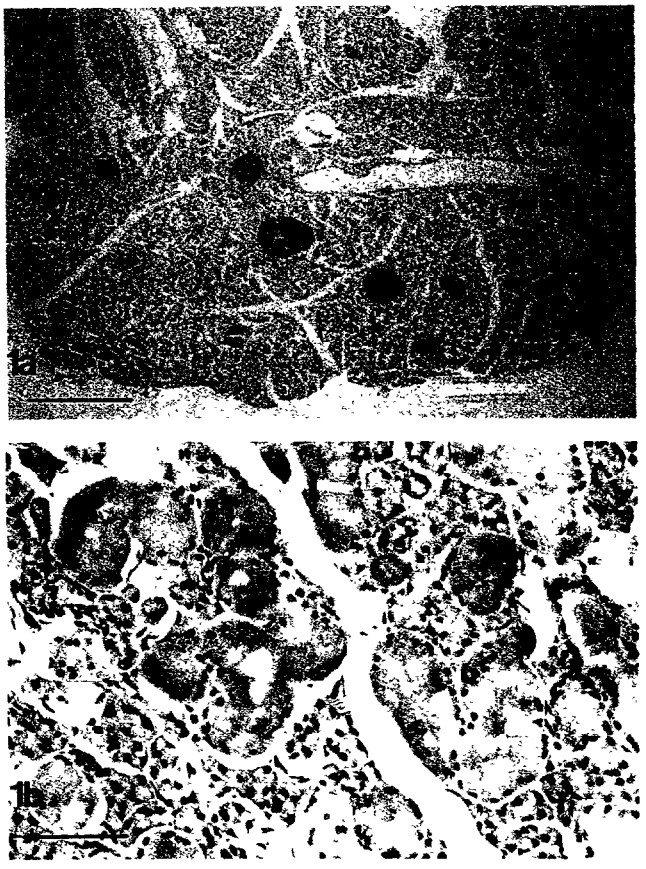

Fig. 1. **a:** Immunolabelling for PrP protein (red) using the 1B3 antibody in a paraffin section of pancreas from a normal uninfected mouse. Labelling is confined to the islets of Langerhans and is found in the same areas in mice infected with scrapie. Scale bar = 500 μm. **b:** Paraffin section of salivary gland from a mouse terminally affected with the 22A strain of scrapie. PrP labelling using 1B3 antibody (brown) is found in mucous secreting cells of the salivary gland. Scale bar 50μm.

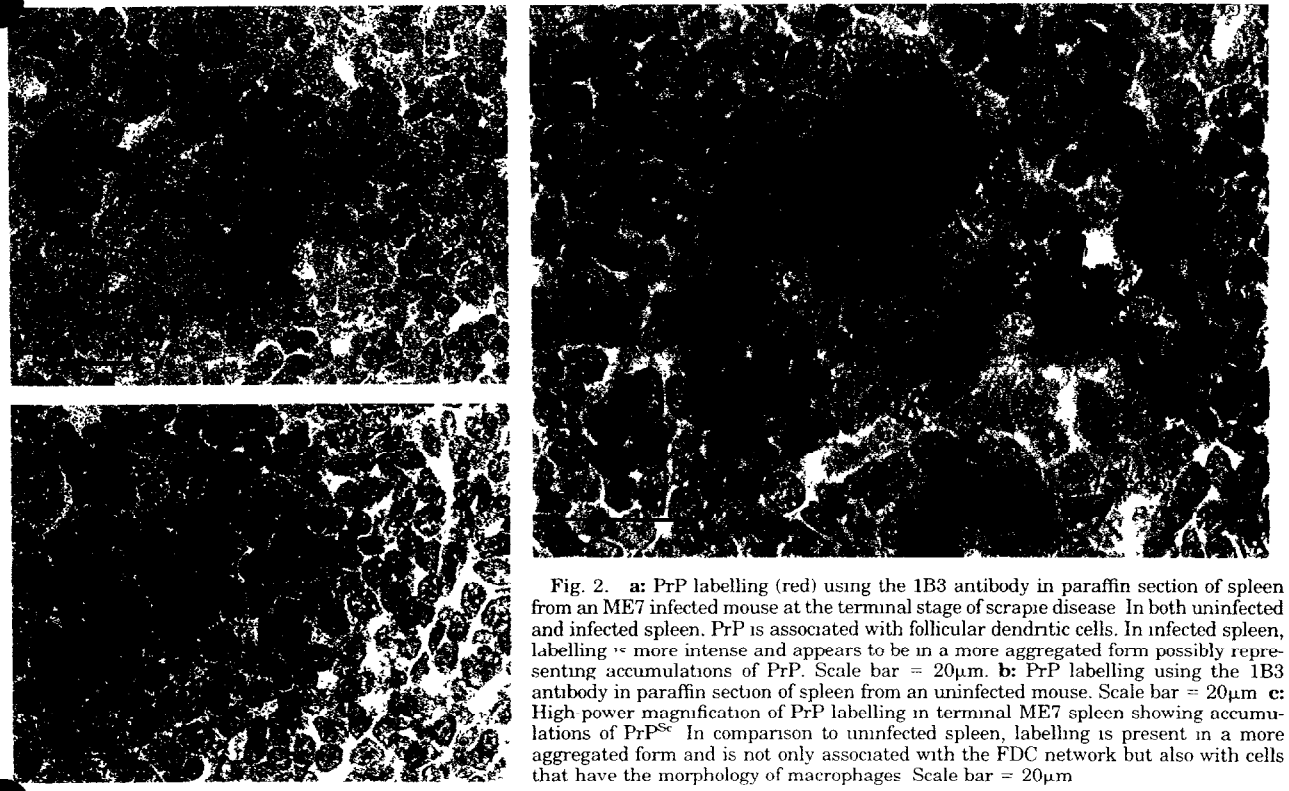

Fig. 2. **a:** PrP labelling (red) using the 1B3 antibody in paraffin section of spleen from an ME7 infected mouse at the terminal stage of scrapie disease In both uninfected and infected spleen, PrP is associated with follicular dendritic cells. In infected spleen, labelling is more intense and appears to be in a more aggregated form possibly representing accumulations of PrP. Scale bar = 20μm. **b:** PrP labelling using the 1B3 antibody in paraffin section of spleen from an uninfected mouse. Scale bar = 20μm **c:** High power magnification of PrP labelling in terminal ME7 spleen showing accumulations of PrP^{Sc} In comparison to uninfected spleen, labelling is present in a more aggregated form and is not only associated with the FDC network but also with cells that have the morphology of macrophages Scale bar = 20μm

Fig. 3. Confocal image of spleen from an ME7 infected mouse at the terminal stage of scrapie disease. Immunolabelling for PrP protein using the 1B3 antibody was carried out on 100-μm-thick vibratome sections. Analysis of PrP immunolabelling using confocal microscopy revealed the presence of immunolabelled cells within germinal centres, which have the morphology of macrophages.

DISTRIBUTION OF PrP PROTEIN IN EXTRANEURAL TISSUES

As PrP protein is essential for disease replication, the identification of the cell types that are associated with PrP in both infected and uninfected mice is important. Although it cannot be assumed that cellular expression of PrP means that these cells participate in replication, it seems likely that the accumulations of PrP^{Sc} in specific cell types indicate an involvement in some aspect of the disease process.

Following infection, PrP^{Sc} accumulates in the CNS although there are dramatic differences in the distribution and appearance of PrP^{Sc} between TSE strains (Bruce et al., 1989). In addition, there are several pathological forms of PrP^{Sc} found within infected brain, which include amyloid plaques, diffuse granular staining of the neuropil, and accumulation around neurons. For example, with the 87V strain of scrapie amyloid plaques are frequent and abnormal accumulations of PrP in brain are precisely targeted to areas of vacuolation, whereas in the ME7 strain of scrapie fewer amyloid plaques are present and PrP pathology is more widely distributed throughout the brain (Bruce et al., 1989).

In spleen and lymph nodes, PrP is associated with FDCs in both uninfected and scrapie infected mice. PrP has also been detected immunocytochemically in several other tissues including, kidney, pancreas, salivary gland, adrenal, liver, and thymus (McBride unpublished) although it is unclear what the function of PrP is in these tissues. In all of these tissues, PrP is localised to specific regions in both uninfected and scrapie infected mice. PrP is found in the cuboidal epithelium of proximal and distal tubules of kidney (not shown), pancreatic islets of Langerhans (Fig. 1a), mucous (but not serous) secreting cells of salivary gland (Fig. 1b), and in the adrenal cortex (not shown), most prominently in the zona fasiculata. Low levels can sometimes be detected in a proportion of liver hepatocytes (not shown). In the thymus, PrP labelling is associated with cells in the thymic medulla (not shown). Although these have not been specifically identified, their morphology and distribution suggests that they may be interdigitating dendritic cells. In these tissues, therefore, PrP is associated with a range of cell types performing different functions, which include secretion, metabolic synthesis, and reabsorption.

PrP IN LYMPHOID TISSUES

In mice, PrP protein is associated with follicular dendritic cells in both uninfected and scrapie challenged animals (McBride et al., 1992). FDCs are non-lymphoid cells, which are found within germinal centres of secondary lymphoid follicles where they trap antigens in the form of antigen antibody complexes.

Whilst PrP on FDCs can be detected immunocytochemically, lymphocytes are also known to express PrP on their cell surface (Cashman et al., 1990; Mabbott et al., 1997) although at lower levels which can only be demonstrated by flow cytometry. Recently, PrP has been detected in lymphoid tissues of sheep with natural scrapie (van-Keulen et al., 1996) and humans with "new-variant" CJD (Hill et al., 1997, 1999).

Although there are no specific antibodies that can discriminate between PrP^{c} and PrP^{Sc} immunocytochemically, the appearance of PrP in infected mice (Fig. 2a) is quite different to that in uninfected mice (Fig. 2b). In spleen from uninfected mice, PrP labelling is very diffuse whereas the labelling in infected mice is much more intense and is not only associated with FDCs but also with cells that have the morphology of macrophages (Fig. 2c). PrP protein also appears to be in a more aggregated form in infected mice, possibly representing accumulations of PrP^{Sc}.

In addition, confocal analysis of spleen from scrapie infected mice has revealed the presence of immunolabelled macrophages, possibly tingible body macrophages, within germinal centres (Fig. 3). Tingible body macrophages are a specialised population of macrophages found within germinal centres. They are mainly

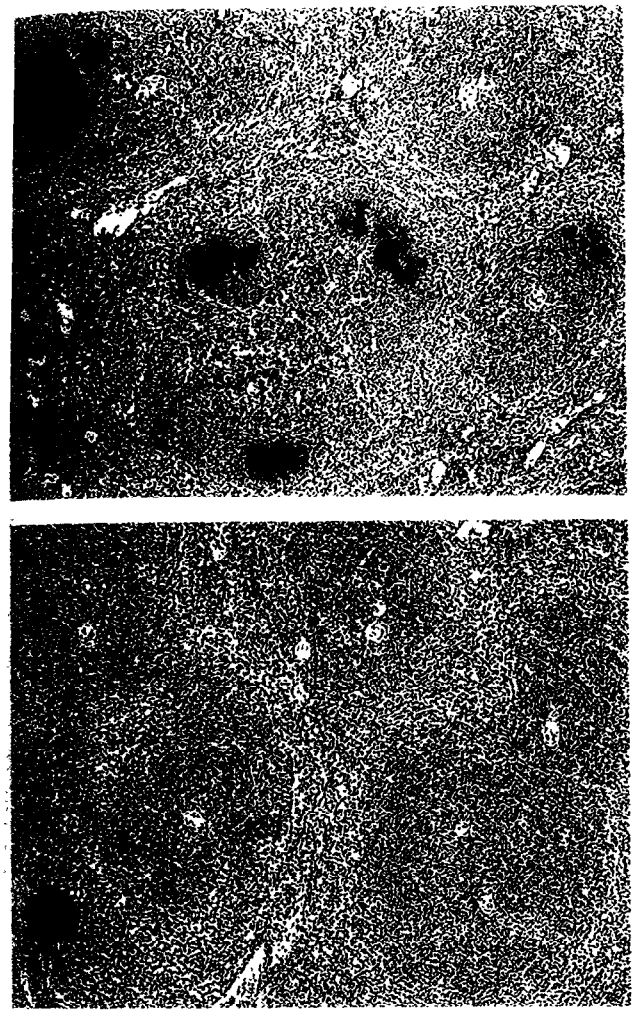

Fig. 4. **a:** Immunolabelling, using the 1B3 antibody, for PrP protein in a terminal ME7 spleen using hydrated autoclaving and formic acid pre-treatment before immunolabelling. Strong PrP labelling can be observed associated with follicular dendritic cells Scale bar = 50 μm **b:** Immunolabelling in an adjacent section to a omitting hydrated autoclaving and formic acid pre-treatment In the absence of these treatments, no labelling for PrP can be observed Scale bar = 50 μm

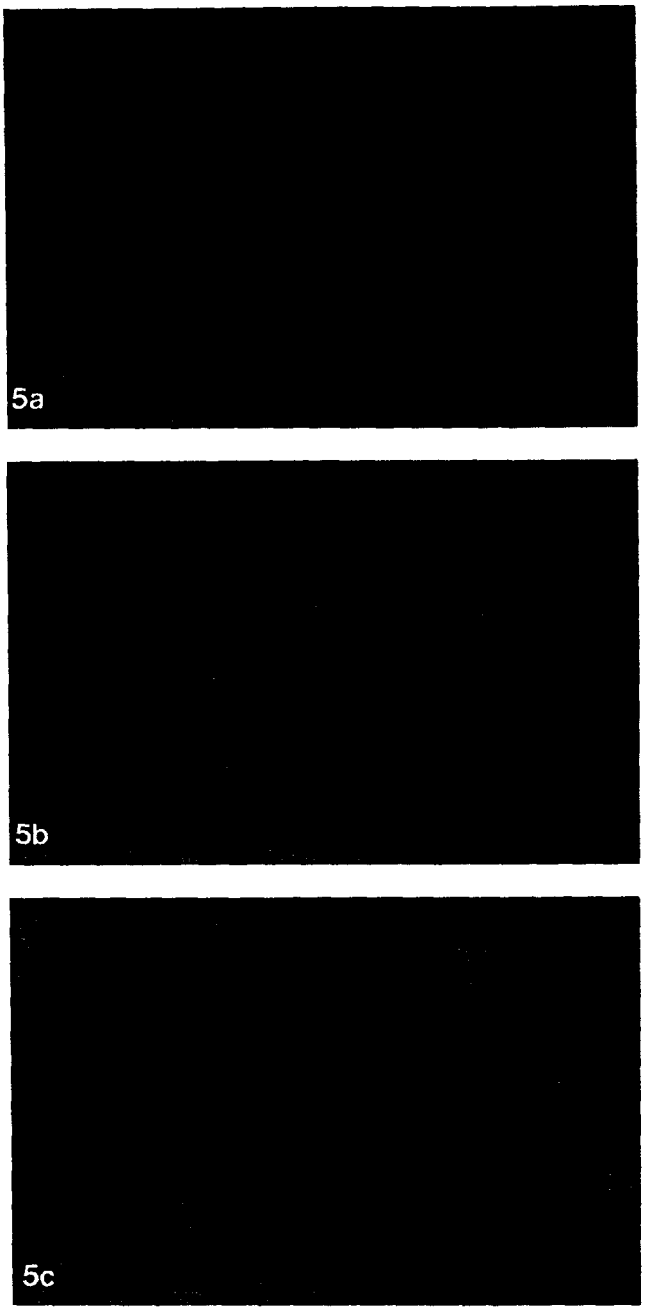

Fig 5. Confocal analysis of double immunoflourescent labelling of (**a**) PrP using the 1B3 antibody (green) or (**b**) FDC networks using the FDC-M1 antibody (red) in 100-μm-thick vibratome sections of spleen from an uninfected mouse Merged confocal image (**c**) (yellow) of the same germinal centre showing co-localisation of PrP and FDCs

involved in the phagocytosis of apoptotic B cells that are not selected for affinity maturation and in the scavenging of FDC processes (Tew et al., 1989).

CURRENT METHODS FOR THE DETECTION OF PrP PROTEIN IN EXTRANEURAL TISSUES

The most widely used methods for detecting PrP protein in tissues are by Western blot or by immunocytochemistry. Although Western blot can be used to distinguish between PrP^{c} and PrP^{Sc}, immunocytochemical analysis can precisely identify which cells are associated with PrP, providing important information on the targeting of infection in disease pathogenesis.

Detection of PrP in Wax-Embedded Tissues

Immunocytochemistry on wax-embedded tissue is probably one of the most useful methods for detection of PrP in tissues from infected mice. Not only is it extremely sensitive but it is also convenient and allows good preservation of tissue morphology. PrP^{c} can be detected in uninfected mice using this method but a newly developed method (Ritchie et al., 1999), which will be discussed later, appears to be more sensitive. Length of fixation is important as over-fixation may lead to reduced intensity of immunostaining (Bruce et

al., 1989; Ritchie et al., 1999). For optimal detection, spleens are normally immersion fixed in periodate-lysine-paraformaldehyde (PLP) fixative for a minimum of 6 hours but no longer than 12 hours. Immunocytochemical visualisation of PrP can be greatly enhanced by the use of pre-treatments. Several methods are currently employed for use on wax sections, which include the use of formic acid treatment (Kitamoto et al., 1987) or hydrated autoclaving (Haritani et al., 1994; Yokoyama et al., 1996) before immunostaining. In addition, hydrated autoclaving followed by formic acid treatment can also be used to enhance PrP immunostaining. All of these pre-treatments will enhance both normal and abnormal forms of PrP in wax-embedded tissues but in the absence of these treatments immunolabelling is greatly reduced or absent (Fig. 4a and b). At present, optimum results for the detection of PrP in spleen have been achieved using the 1B3 polyclonal antibody (Farquhar et al., 1989) and the avidin-biotin or streptavidin- biotin technique or the peroxidase anti-peroxidase technique (PAP). Recently, we have employed the use of fluorescent conjugated antibody substrates such as FITC or Cy3™ with encouraging results.

Visualisation of PrP and FDCs Within the Same Tissue Section

PrP protein in infected mice is detected optimally using wax-embedded tissue sections. However, attempts to use the murine specific antibody to FDCs (FDC-M1) on PLP wax-embedded spleen have been unsuccessful (Brown, unpublished data). FDC-M1 works well on acetone-fixed tissue sections but immunolabelling for PrP protein in infected mice is less intense than in wax-sections, possibly because pre-treatments such as formic acid cannot be used with this method of fixation.

Recently, new methods have been developed (Ritchie et al., 1999), which allow good immunolabelling of both PrP and FDCs within the same tissue section. Briefly, mice were perfused with PLP and spleens post-fixed in PLP for 4 hours. In developing this method, the length of fixation was found to be critical as over-fixation reduced labelling of PrP and FDCs. Following fixation, spleens were placed in phosphate buffer and 100-μm sections of spleen cut using a vibratome. For PrP labelling, 1B3 antibody was applied to free-floating sections overnight and visualised using an FITC conjugated goat anti-rabbit antibody (Fig. 5a). For detection of FDCs, FDC-M1 was applied to adjacent sections for 1 hour and visualised using streptavidin conjugated Cy3™ (Fig. 5b). In the development of this technique, formic acid was used as a pre-treatment for spleen from both normal and scrapie-infected spleens. Interestingly, formic acid had little effect on the appearance or intensity of PrP in spleen from normal mice but greatly enhanced the appearance and intensity of PrP in infected spleen. In the absence of formic acid pre-treatment, very little PrP could be demonstrated in scrapie-infected spleen suggesting that formic acid pre-treatment may be useful in discriminating between normal and abnormal forms of the protein. In addition, formic acid pre-treatment of scrapie-infected spleens revealed the presence of immunolabelled cells with the morphology of macrophages that were unlabelled when formic acid treatment was omitted. Although the mechanism by which formic acid enhances PrP^{Sc} is not understood, one explanation is that it may destroy cell membranes allowing the visualisation of PrP within cells (for example PrP ingested by tingible body macrophages). Alternatively, it may reveal epitopes that are hidden in the aggregated forms of PrP that accumulate following infection.

For double immunolabelling, 1B3 was used as the first stage antibody using the same procedures as described for single immunolabelling. However, the development of double immunolabelling was problematic in scrapie-infected mice. Formic acid pre-treatment was found to reduce FDC labelling and even removed staining from sections that were pre-labelled with FDC-M1. This may suggest that the specific antigen recognised by FDC-M1 is altered or removed by formic acid treatment possibly because of cell membrane disruption. As a result, double immunolabelling was not possible in spleens from scrapie-infected mice. However, successful double labelling of uninfected spleen (Fig. 5c) quite clearly demonstrated the co-localisation between PrP and FDCs when viewed by confocal microscopy (Biorad MRC 500).

CONCLUSION

Although biochemical methods can distinguish between abnormal and normal forms of PrP in extraneural tissues, immunocytochemical methods are undoubtedly extremely useful for investigating the association of PrP with particular cell types. As the peripheral lymphoid system clearly plays an important role in TSE infection, immunocytochemistry at both the light and ultrastructural level will be useful in investigating early changes in these tissues and in identifying potential targets cells for prophylaxis and therapy.

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